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# Specificity of the second binding protein of the peptide ABC-transporter (Dpp) of *Lactococcus lactis* IL1403

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## Abstract

The genome sequence of *Lactococcus lactis* IL1403 revealed the presence of a putative peptide-binding protein-dependent ABC-transporter (Dpp). The genes for two peptide-binding proteins (*dppA* and *dppP*) precede the membrane components, which include two transmembrane protein genes (*dppB* and *dppC*) and two ATP-binding protein genes (*dppD* and *dppF*). In this work, the gene specifying the second peptide-binding protein (DppP) was cloned under the control of the nisin promoter. The protein fused to a carboxyl-terminal histidine tag (DppP-His<sub>6</sub>) was purified and its binding properties were determined by monitoring the intrinsic fluorescence changes observed upon ligand binding. The major features of peptide binding to DppP-His<sub>6</sub> include: (i) a requirement for a free N-terminal  $\alpha$ -amino group in the ligand; (ii) a high affinity for di-, tri- and tetra-peptides; (iii) affinity constants for peptide binding independent of pH; and (iv) a high affinity for D-isomer-containing peptides. Remarkably, the features (ii), (iii) and (iv) differ from those previously reported for DppA-His<sub>6</sub>, suggesting that DppP-His<sub>6</sub> is a more versatile peptide-binding protein that could have additional functions.

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**Keywords:** Peptide-binding protein; ABC-transporter; *Lactococcus lactis*

## 1. Introduction

In bacteria, peptide transporters serve a role in nutrition by supplying exogenous substrates as nitrogen and carbon sources, but the systems can also function in regulation of gene expression, chemotaxis, conjugation and/or competence development [1–3]. In most cases, peptide transport is mediated by solute-binding protein-dependent ABC-transporters. These are multicomponent systems composed of two integral membrane subunits, two peripheral ATP-binding subunits and an extracytoplasmic solute-binding receptor. In general, the solute-binding proteins provide the primary interaction site for the ligand and largely define the specificity of the transport system [4].

*Lactococcus lactis* possesses at least three peptide transport systems, one specific for oligopeptides (Opp) and two others for di- and tri-peptides (DtpT and Dpp, the latter

has been previously referred to as DtpP; [4]). The proteolytic pathway of *L. lactis* is initiated by the degradation of caseins by an extracellular proteinase resulting in the generation of oligopeptides from 4 to 30 residues, most of which are substrates of Opp [6–9]. Whereas Opp is considered essential for nutrition (accumulation of essential amino acids, present in the oligopeptides), the di-tri-peptide transport systems are thought to serve alternative roles, like regulation of expression of genes involved in nitrogen metabolism [10–13]. The presence of a second di-tripeptide transport system (Dpp), in addition to the firstly characterized DtpT, was initially deduced from the ability of *opp/dtpT* double mutants to transport hydrophobic di- and tri-peptides [5]. Recently, the genes encoding a putative peptide-binding protein-dependent ABC-transporter (*dpp*) were identified in *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1343 [13,14]. In both strains the *dpp* system has two possible peptide-binding protein genes (*dppA* and *dppP*), which precede the genes for the membrane complex (*dppBCDF*). The expression of more than one binding protein could allow the system to transport a wider range of peptides.

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Functional studies of the Dpp system from *L. lactis* were initiated in the strain MG1363 since its proteolytic system is the best characterized to date. However, we found that in *L. lactis* MG1363 only *dppA* encodes a functional di-tripeptide-binding protein, while *dppP* contains a chain-terminating non-sense mutation that may impair its function [13,15]. In contrast, in *L. lactis* IL1403 both homologous genes seem to encode functional peptide-binding proteins that could have evolved to play different physiological roles. Despite the fact that only few residues define the binding properties of solute-binding proteins, alignments of the deduced amino acid sequences of DppA and DppP show enough differences (55% identity) to anticipate variations in specificity.

In this work, we report on the purification and specificity of the second peptide-binding protein (DppP) of the Dpp system from *L. lactis* IL1403. The binding properties are compared to those previously reported for DppA and OppA, revealing important differences between these systems [8,9,15].

## 2. Materials and methods

### 2.1. Strains and growth conditions

The strain *L. lactis* NZ9000 (MG1363 derivative, *pepN::nisRK*) was kindly provided by O. Kuipers and used to overexpress the *dppP* gene of *L. lactis* IL1403 [16] after transformation with the vector pNZDppP. The supernatant of cultures of *L. lactis* NZ9700 [17] was used as a source of nisin A to trigger the transcription from the *nisA* promoter. The strains were grown, at 30°C, in M17 broth or agar (Difco, East Molesey, UK) supplemented with 0.5% (w/v) glucose and 5 µg ml<sup>-1</sup> of chloramphenicol, when appropriate.

### 2.2. Plasmid construction and general DNA manipulations

For the construction of pNZDppP, the gene (*dppP*) encoding the peptide-binding protein of the Dpp system was amplified from *L. lactis* IL1403 genomic DNA with Vent DNA polymerase (New England BioLabs, Beverly, MA, USA). In the forward primer (5'-CGCGCCATGGATAGCGGTTCTAGCTCATCG-3'), a *NcoI* restriction site (underlined) was introduced 3' of the sequence encoding the signal peptide. In the reverse primer (5'-GGCGGATCCTTTGAGGTAAGCTGTTTTG-3'), a *BamHI* restriction site (underlined) was created that overlaps with the stop codon of the *dppP* gene. The polymerase chain reaction (PCR) product was digested with *NcoI* and *BamHI* and ligated with the 3.7-kb fragment obtained by digestion of pHLP5 [18] with the same restriction enzymes. The resultant plasmid, pNZDppP, carries the *dppP* gene cloned under the control of the *nisA* promoter and in frame with the sequence specifying a carboxyl-

terminal factor Xa cleavage site and a six-histidine tag [18].

Molecular cloning techniques were performed essentially as described by Sambrook et al. [19]. Plasmid DNA from *L. lactis* was isolated by either the method of Birnboim and Doly, with minor modifications [20], or the Qiagen column purification kit (Qiagen, GmbH, Hilden, Germany). PCR was performed with Vent DNA polymerase according to the instructions of the supplier (New England BioLabs, Beverly, MA, USA). *L. lactis* was transformed by electroporation as described by Holo and Nes [21].

### 2.3. Expression and purification of DppP-(His)<sub>6</sub>

The expression of DppP-(His)<sub>6</sub> was induced with nisin A, as described elsewhere [15]. Briefly, *L. lactis* NZ9000, carrying the vector pNZDppP, was grown to an OD<sub>660nm</sub> of approximately 1.0. At that stage, the expression of DppP-(His)<sub>6</sub> was triggered by the addition of 1:1000 dilution of the filtered supernatant of a *L. lactis* NZ9700 culture (containing about 10 ng of nisin A ml<sup>-1</sup>). The induction time was 2 h, and at the end of this period the cells had reached a final OD<sub>660nm</sub> of around 2.6. Cells were harvested by centrifugation at 6000×g for 10 min at 4°C, washed twice in 50 mM sodium phosphate, pH 7.0, and suspended in the same buffer, supplemented with 20 mM imidazole and 100 mM NaCl. Cells were disrupted with glass beads in a Bead-beater (Biospec Products, Bartlesville, OK, USA) and the cell-free extract was recovered by centrifugation (12 000×g, 10 min, at 4°C). The protein was purified from the supernatant by two chromatographic steps. First, the extract was loaded onto a Bio Spin column (Bio-Rad, Hercules, CA, USA) containing Ni<sup>2+</sup>-NTA resin (Qiagen, GmbH, Hilden, Germany). The washing step was carried out with 50 mM sodium phosphate, pH 6.5, containing 50 mM imidazole and the elution step was carried out with the same buffer containing 200 mM imidazole. Final purification of DppP-(His)<sub>6</sub> was achieved by cation-exchange chromatography on a Resource S column (1 ml; Pharmacia). Proteins were eluted at 1 ml min<sup>-1</sup> by applying an initial isocratic step in the equilibration buffer (50 mM sodium phosphate, pH 6.0), followed by a linear gradient from 0 to 300 mM NaCl, in the same buffer.

The endogenous ligand possibly present in purified DppP-His<sub>6</sub> was eliminated by partial denaturation with guanidine hydrochloride and renaturation of the protein as previously described [8,15].

### 2.4. Protein concentration

Protein concentration was determined by the bicinchoninic acid (BCA) method with the BCA protein assay reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as the standard. The concentration of the purified protein was also estimated from the optical den-

sity at 280 nm, using a calculated extinction coefficient of  $1.315 \text{ (mg ml}^{-1}\text{)}^{-1} \text{ cm}^{-1}$ .

### 2.5. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [22], using 6% acrylamide stacking gels and either 10 or 12% acrylamide resolving gels. Proteins were visualized by Coomassie brilliant blue staining.

### 2.6. Fluorometric assays

Fluorescence spectra were obtained on a spectrofluorometer Shimadzu RF-500 (Kyoto, Japan), at 20 °C. Excitation was at 278 nm and emission was scanned from 200 to 400 nm using 5-nm bandwidths. Titration of intrinsic fluorescence of DppA-(His)<sub>6</sub> with different ligand concentrations was performed by excitation at 278 nm, using a 3-nm bandwidth, and by monitoring the emission at 340 nm, using a 10-nm bandwidth. Ligand binding studies were generally performed in 50 mM sodium phosphate, pH 6.5. When ligand binding was analyzed as a function of pH, the following buffers were used: 50 mM sodium-acetate/acetic acid (pH 4.5–5.5) and 50 mM sodium phosphate (pH 6.0–7.5). Binding curves were analyzed according to a hyperbolic binding equation, as described elsewhere [8,15]. Non-linear least-squares regression was performed with the Sigma Plot program (Jandel Scientific Software). The estimated binding parameters are the average of three independent determinations and the standard deviations are given.

## 3. Results and discussion

### 3.1. Amplified expression and purification of DppP-(His)<sub>6</sub>

The synthesis of the protein DppP-(His)<sub>6</sub> was amplified by the use of the nisin-controlled expression system, which utilizes the antimicrobial peptide nisin as inducer [23]. The gene *dppP* was translationally fused to the *nisA* promoter without the sequence specifying the signal peptide and fused to that encoding a carboxyl-terminal six-histidine tag, as described elsewhere [13]. Thus, the peptide-binding protein was expressed as a soluble protein carrying a histidine tag [DppP-(His)<sub>6</sub>], which altogether facilitates the purification and characterization procedures. The purification to homogeneity was achieved by two consecutive chromatographic steps, which included Ni<sup>2+</sup> affinity chromatography (Ni<sup>2+</sup>-NTA resin) and cationic chromatography (Resource-S). From the first chromatographic step the peptide-binding protein eluted at 200 mM imidazole, achieving about 80–85% purity (Fig. 1, lane 3). From the second chromatographic separation on the strong cation-exchange column the target protein eluted at 255 mM

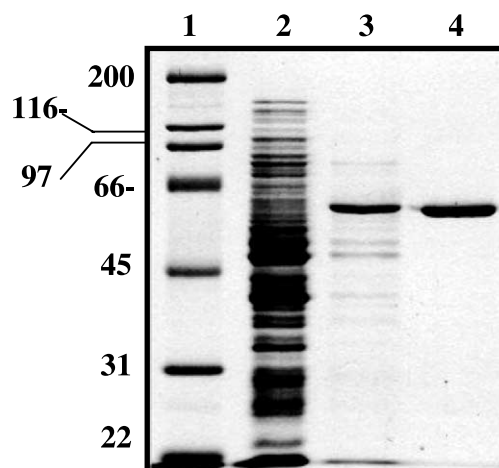


Fig. 1. SDS–PAGE analysis of DppP-(His)<sub>6</sub> purification. Lane 1, molecular mass markers (kDa); lane 2, cell-free extract; lane 3, samples eluted from Ni<sup>2+</sup>-NTA chromatography at 200 mM imidazole; lane 4, purified protein eluted from cation-exchange chromatography at 255 mM NaCl.

NaCl while most of the contaminant proteins eluted in the isocratic step. A highly purified and homogeneous sample with an expected molecular mass of 58 kDa was obtained, as shown by SDS–PAGE (Fig. 1). The procedure yielded about 1.4 mg of purified protein l<sup>-1</sup> of cell culture of OD<sub>660nm</sub> of 2.6. The endogenous ligand possibly present in purified DppP-His<sub>6</sub> was eliminated by partial denaturation of the protein bound to the Ni<sup>2+</sup>-NTA resin with 3 M guanidine hydrochloride. The renaturation of the protein was achieved by applying successive washing steps with decreasing concentrations of guanidine hydrochloride from 2.5 to 0 M [15]. About 60% of purified protein was recovered after the denaturation–renaturation procedure.

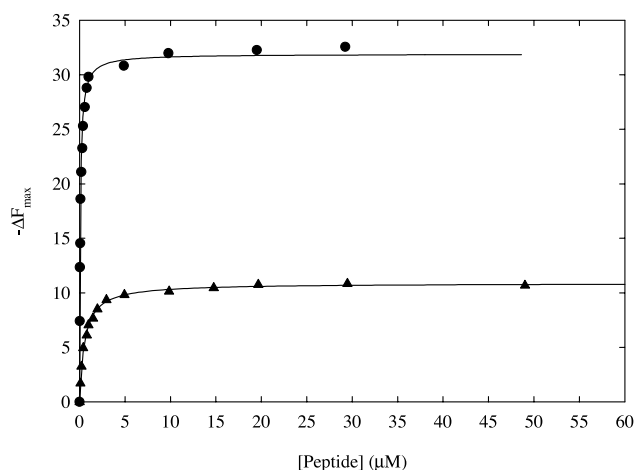


Fig. 2. Fluorescence titration with various peptides. Intrinsic protein fluorescence from 0.025 μM of DppP-(His)<sub>6</sub> in 50 mM sodium phosphate, pH 6.5, upon addition of increasing concentrations of Leu-Leu (▲) and Leu-Leu-Leu (●). The solid line represents the best fits of the data to a hyperbolic binding equation [8,15].

### 3.2. Specificity and binding affinities of DppP-(His)<sub>6</sub> estimated from changes in the intrinsic protein fluorescence.

The binding mechanism of ABC-transporter-associated solute-binding proteins involves a conformational change that can be monitored by different techniques. Peptide binding to OppA and DppA from *L. lactis* MG1363 was previously shown to elicit a change in the intrinsic protein fluorescence that allows the accurate determination of the kinetics of peptide binding [8,15]. The intrinsic protein fluorescence of DppP-His<sub>6</sub> was determined, showing excitation and emission maxima at 278 and 340 nm, respectively (data not shown). The addition of saturating concentrations of ligand resulted in a blue shift in the emission maxima and in an overall decrease in fluorescence from 13 to 30% depending on the peptide. The decrease in fluorescence at 340 nm with increasing concentrations of ligand was used to determine the kinetic parameters of peptide binding to DppP-His<sub>6</sub> (0.05–0.025  $\mu$ M). The data of fluorescence changes as a function of peptide concentration were fitted to a hyperbolic single-site model, as described elsewhere [15] (Fig. 2). Initially, the pH dependence of binding was determined using Ala-Ala as ligand. As shown in Fig. 3, only moderate changes in affinity constants ( $K_d$  varied from 0.38 to 0.68  $\mu$ M) were observed in the pH range from 4.5 to 7.5. DppP-His<sub>6</sub> showed the highest affinity at pH 6.5, slightly decreasing beyond this value. In contrast, the affinity of peptide binding to DppA-His<sub>6</sub> was greatly affected by pH, that is, the  $K_d$  decreased about one order of magnitude per unit increase in pH above the pH of 6.0 [15].

Structural information of peptide-binding proteins has been determined from X-ray crystallographic studies of DppA from *Escherichia coli* and OppA from *Salmonella typhimurium* [24–27]. The binding mechanism is considered

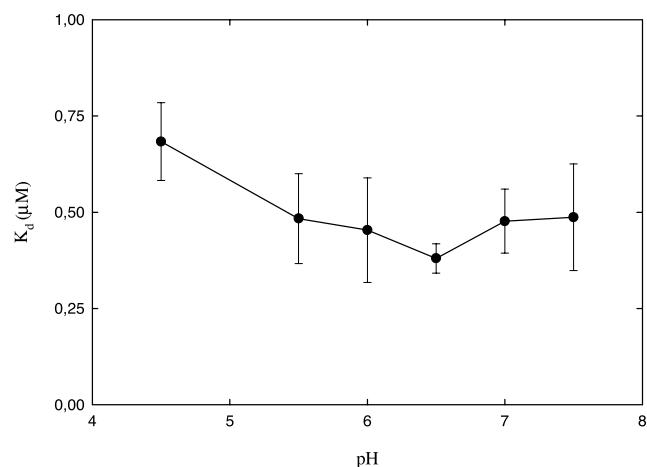


Fig. 3. pH dependence of dissociation constants ( $K_d$   $\mu$ M) for peptide binding to DppP-(His)<sub>6</sub>. Binding saturation curves were obtained for Ala-Ala at different pH values (4.5–7.5) with 0.05  $\mu$ M of DppP-(His)<sub>6</sub>; the binding parameters were calculated from the corresponding fits of the data.

Table 1

Effect of amino-acyl side chain and ligand length on peptide binding<sup>a</sup> (mean  $\pm$  S.D.,  $N = 3$ )

Peptide	$\Delta F_{\max}$ (%)	$K_d$ ( $\mu$ M)
Val-Val	21.2 $\pm$ 6.5	0.44 $\pm$ 0.02
Leu-Leu	13.8 $\pm$ 2.9	0.49 $\pm$ 0.11
Ala-Ala	13.3 $\pm$ 1.4	0.38 $\pm$ 0.04
Gly-Gly	19.7 $\pm$ 1.2	0.45 $\pm$ 0.02
Asp-Asp	18.5 $\pm$ 3.3	0.49 $\pm$ 0.08
Glu-Glu	13.1 $\pm$ 2.7	0.71 $\pm$ 0.08
Lys-Lys	14.7 $\pm$ 2.3	0.49 $\pm$ 0.13
Leu-Leu-Leu	30.8 $\pm$ 1.2	0.09 $\pm$ 0.01
Ala-Ala-Ala	26.8 $\pm$ 1.0	0.09 $\pm$ 0.01
Lys-Lys-Lys	25.5 $\pm$ 1.8	0.07 $\pm$ 0.01
Ala-Ala-Ala-Ala	23.1 $\pm$ 4.2	0.38 $\pm$ 0.02
Val-Ala-Pro-Gly	16.6 $\pm$ 4.9	0.72 $\pm$ 0.09
Gly-Arg-Gly-Asp	24.6 $\pm$ 3.3	0.85 $\pm$ 0.11
Gly-His-Arg-Pro	30.7 $\pm$ 0.8	0.43 $\pm$ 0.06
Gly-Pro-Gly-Gly	23.8 $\pm$ 1.4	0.39 $\pm$ 0.03

<sup>a</sup>Binding parameters were estimated from the quenching of the intrinsic protein fluorescence at 340 nm upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.5, at 20°C.

to be by the so-called Venus flytrap principle, in which the peptide is engulfed inside the binding protein, with the N- and C-termini of the peptide interacting with conserved charged amino acids in the binding protein. The peptide-binding mechanism of OppA from *L. lactis* differs from that of the two enteric bacteria proteins, which allows the Opp system to transport much longer peptides, up to at least 18 residues [3,6,8]. In the X-ray structures of DppA from *E. coli* and OppA from *S. typhimurium*, the amino acid side chains are projected in spacious and hydrated pockets in which few direct contacts are made with the protein. The nature of the amino-acyl side chain exerted little effects on the binding properties of DppA-His<sub>6</sub> (Table 1). Even the  $K_d$  for Gly-Gly, which has been described as a poor substrate for all characterized di-peptide-binding proteins, was similar to those obtained for the other tested di-peptides (Table 1). DppP-His<sub>6</sub> requires a free N-terminal  $\alpha$ -amino group but the protein does not have a strong requirement for a free C-terminal  $\alpha$ -carboxyl group as demonstrated with *N*-acetylated and *O*-methylated di-alanine (Table 2). The removal of the positive charge from

Table 2

Stereospecificity and effect of peptide modification on peptide binding<sup>a</sup> (mean  $\pm$  S.D.,  $N = 3$ )

Peptide	$\Delta F_{\max}$ (%)	$K_d$ ( $\mu$ M)
L-Ala-L-Ala	13.3 $\pm$ 1.4	0.38 $\pm$ 0.04
D-Ala-L-Ala	20.8 $\pm$ 1.9	0.48 $\pm$ 0.11
L-Ala-D-Ala	19.3 $\pm$ 2.3	0.51 $\pm$ 0.03
D-Ala-D-Ala	20.6 $\pm$ 1.1	0.41 $\pm$ 0.04
Ac-Ala-Ala	nd <sup>b</sup>	nd
Ala-Ala-OMe	17.1 $\pm$ 0.6	0.59 $\pm$ 0.03

<sup>a</sup>Binding parameters were estimated from the quenching of the intrinsic protein fluorescence at 340 nm upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.5, at 20°C.

<sup>b</sup>nd, binding not detected by fluorescence spectroscopy up to 100  $\mu$ M.



Table 3  
Specificity of DppP-(His)<sub>6</sub> for dipeptides<sup>a</sup> (mean ± S.D., N = 3)

Peptide	$\Delta F_{\max}$ (%)	$K_d$ (μM)
Ala-Met	22.1 ± 0.4	0.43 ± 0.04
Met-Ala	21.5 ± 1.1	0.53 ± 0.05
Ala-Asp	15.5 ± 3.0	0.37 ± 0.06
Asp-Ala	17.2 ± 5.5	0.49 ± 0.07
Ala-Lys	19.2 ± 0.4	0.57 ± 0.11
Lys-Ala	14.7 ± 2.3	0.31 ± 0.05

<sup>a</sup>Binding parameters were estimated from the quenching of the intrinsic protein fluorescence at 340 nm upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.5, at 20°C.

the N-terminal  $\alpha$ -amino group in the *N*-acetyl-derivatives abolished ligand binding to all the peptide-binding proteins studied to date, except for OppA. A subset of peptide-binding proteins, but not OppA, has a conserved aspartic acid that interacts with the positively charged  $\alpha$ -amino group of the peptide [15,28]. This residue is also conserved in DppP from *L. lactis* (data not shown) consistent with the prominent effect of peptide acetylation on binding to the protein. The mechanism of oligopeptide binding to OppA from *L. lactis* constitutes an exception since only the first six amino acids of the peptide are enclosed in the binding pocket, whereas the remaining residues interact with the protein surface [9]. Thus, the interaction of OppA with the N- and C-termini of the peptide is not as critical as in the other peptide-binding proteins [7].

The effects of the length of the peptide on binding affinities were studied for di-, tri- and tetra-peptides. DppP-His<sub>6</sub> showed a preference for hydrophobic tri-peptides, lowest  $K_d$  and highest  $\Delta F_{\max}$  values, when compared with the corresponding di-peptides (Table 1).

Remarkably, DppP-His<sub>6</sub> showed a high affinity for tetra-peptides (Table 1). Binding of tetra-peptides was not observed for DppA or DtpT, and transport of these peptides was attributed to Opp only [15,29,30]. According to the presented results a role for Dpp from *L. lactis* I11403 in the uptake of casein-derived peptides needs to be considered. *L. lactis* MG1363 may not transport tetra-peptides, when *opp* is deleted, because the corresponding DppP protein of the Dpp system is inactive but the situation may be different in *L. lactis* IL1403 [31]. According to studies of peptide transport in *E. coli*, *Bacillus subtilis* and *Streptococcus*, the corresponding Dpp systems seem to be specific for the uptake of only di-peptides and to a lesser extent tri-peptides [32–34].

A limited number of substrates were tested to determine the effect of the position of the amino acid residues on binding properties but, in every case, the affinities were not greatly affected. The presence of a basic residue at the N-terminus of the di-peptide Lys-Ala caused a moderate increase in affinity when compared with that of Ala-Lys (Table 3). DppA from *E. coli* and *L. lactis* show stereochemical preference for L-residues, which matches

the di-peptide transport specificity of Dpp in *E. coli* [28]. Moreover, the presence of D-Ala residues did not provoke a drastic reduction in affinity in the case of DppP-His<sub>6</sub> of *L. lactis* (Table 2). In the light of these results and in analogy with the situation in *E. coli*, it is possible that DppP-His<sub>6</sub> plays a role in recycling of cell wall peptides. In *E. coli* a specific peptide-binding protein, MppA, is required for the import of murein tri-peptides [34].

In conclusion, DppP-His<sub>6</sub> seems to be a more versatile peptide-binding protein, with broader specificity, less strict structural requirements and lower affinities (higher  $K_d$  values) than DppA-His<sub>6</sub> from *L. lactis* and the homologous proteins in the enteric bacteria. The binding properties presented here contribute to a better understanding of the proteolytic system of *L. lactis* as the presence of a broad-specificity binding protein next to OppA was not foreseen on the basis of earlier work.

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